SUPPLEMENTAL MATERIAL

Similarity of Bisphenol A Pharmacokinetics in Rhesus Monkeys and Mice: Relevance for Human Exposure

Julia A. Taylor[1], Frederick S. vom Saal[1], Wade V. Welshons[2], Bertram Drury[1],

George Rottinghaus[3], Patricia A. Hunt[4], and Catherine A. VandeVoort[5]

[1]Division of Biological Sciences
[2]Department of Biomedical Sciences
[3]Veterinary Medical Diagnostic Laboratory
University of Missouri
Columbia, MO

[4]School of Molecular Biosciences
Washington State University
Pullman, WA

[5]Department of Obstetrics and Gynecology
California National Primate Research Center
University of California
Davis, California

Running Title: Comparison of BPA Kinetics in Mice, Monkeys and Humans.

Key words: biomonitoring, bisphenol A, endocrine disruption, pharmacokinetics, xenobiotic metabolism.

Communicating Author
Julia A. Taylor
Division of Biological Sciences
205 Lefevre Hall
University of Missouri
Columbia, MO 65211
TEL: 573-882-2482
FAX: 573-884-5020
EMAIL: taylorja@missouri.edu

CHEMICALS

Tritiated BPA (^{3}H-BPA; specific activity 7.3 Ci/mmol) was obtained from Moravek Biochemicals (Brea, CA), and unlabeled BPA (>99% pure) was obtained from Aldrich (Milwaukee, WI). Tocopherol-stripped corn oil was from MP Biomedicals (Solon, OH). Methanol, water and tert-butyl methyl ether were HPLC grade and obtained from Fisher Scientific (Pittsburgh, PA). Deuterated (d6)-BPA was purchased from C/D/N Isotopes Inc. (Pointe-Claire, Quebec, Canada).

Water used in these studies was tested for the presence of background BPA, after concentration on C18 Sep-paks (see Experiment 2C, below). BPA was not detected in this water, even after a 250-fold concentration. Other potential sources of laboratory water tested had values of 0-0.16 ng/ml. We have no evidence for BPA leaching from the HPLC equipment or solvents; blank samples did not appear to contain BPA, and spiked samples gave anticipated values.

EXPERIMENT 1

Deuterated BPA (dBPA) administration and sample collection for monkeys. All animals were trained to accept small pieces of fruit prior to beginning the dBPA treatment period. Fruit was small enough that animals would take the fruit in one bite and did not try to pull it into smaller pieces prior to consuming it. Preferences of each animal were noted. The dBPA dose for each animal was calculated based on body weight the day before the treatment period began. dBPA was prepared as a 25 mg/ml ethanol stock solution, and the daily dose fed was 400-µg/kg body weight given daily in the morning for 7 days. The dBPA/ethanol solution (100-150 µl) was injected with a Hamilton 200 µl syringe into the center of fruit pieces, such as grapes, banana slices, dates or dried apricots, so that the animal could grasp the fruit and place it in its mouth without touching the dBPA.

Isotope dilution LC-MS analysis of unconjugated and conjugated dBPA. Serum samples (1-2 ml) were spiked with ^{13}C-BPA (Cambridge Isotopes Laboratories, Andover, MA) as an internal standard, and extracted twice with methyl tert-butyl ether for determination of unconjugated dBPA. The ether extract was dried under nitrogen and reconstituted in 60:40 methanol:water. After extraction of unconjugated dBPA, for analysis of unextracted conjugated dBPA (glucuronidated and sulfated forms), the samples were treated overnight at 37^{o}C with b-glucuronidase/aryl sulfatase (Sigma) and then extracted by the same procedure used for unconjugated dBPA.

Serum dBPA was assayed by LC-MS using a Thermo Finnigan Surveyor MSQ plus connected to an integrated Thermo-Accela LC system; analytes were detected using electrospray ionization with negative polarity, a cone voltage of 70V, and probe temperature of 600^{o}C. Separations were performed on a 1.9 micron Hypersil Gold HPLC column (50x2.1 mm) with a mobile phase gradient running from 20% to 95% acetonitrile over 6 minutes, at 550 µl/minute. dBPA and ^{13}C-BPA were detected using selected ion monitoring for m/z 233 and m/z 239 respectively. Thermo Xcalibur software was used to autotune, acquire, and process the LC/MS data. Isotope dilution quantitation was made against a standard curve of at least 5 calibration standards (dBPA and ^{13}C-BPA) to adequately cover the expected BPA concentration range. The limit of detection (LOD) for BPA in serum was 0.2 ng/ml (parts per billion, ppb) based on extraction of 2 ml of serum.

EXPERIMENT 2-A

Methods for measuring unconjugated ^{3}H-BPA in serum. Two volumes of cold absolute methanol were added to volumes of serum ranging from 150-350 ml. Precipitated proteins were pelleted at 4^{o}C by centrifugation for 15 minutes at 3,000 x *g*. The supernatant was dried under nitrogen, and brought to 50% methanol by the addition of 75 ml methanol and 75 ml distilled deionized H_2O. The

reconstituted samples were separated by HPLC on a reverse phase Hypersil C18 column (4.6 x 100 mm, Phenomenex), using a mobile phase of 65% methanol at a flow rate of 0.55 ml/min, as previously described (Taylor et al. 2008). Elution of separated components was monitored by UV absorbance at 260 nm on a Perkin-Elmer LC-90 spectrophotometric detector, and also using a bRAM in-line scintillation counter (IN/US Systems, FL) to monitor radioactivity. Authentic ^{3}H-BPA (Moravek) was used as a standard to identify expected elution times. Fractions from injected samples were collected at 20-second intervals across a window spanning the authentic BPA elution time, and radioactivity per fraction was counted on a scintillation counter for 10 minutes/sample (this provides greater sensitivity and accuracy than the bRAM measurements).

BPA was quantified by summing the radioactivity in the fractions eluting at the same time points as authentic BPA. Counts per minute (cpm) were converted to mass by referencing the specific activity of the original administered oil sample. The sensitivity of the assay, calculated as two-fold above background cpm, was 0.28 ng BPA/ml serum.

The running time for BPA was verified at regular intervals using ^{3}H-BPA and also using positive control samples, which consisted of untreated mouse serum containing ^{3}H-BPA (~2700 cpm per 100 µl). The recovery of the added ^{3}H-BPA, determined by comparing the sum of the radioactivity measured in the HPLC fractions to radioactivity in spiked plasma that had not been extracted, averaged (±SEM) 84.1±10.4% across 4 positive control sample runs. Background counts, determined individually for all sample runs were similar, averaging 13.17±0.868 cpm. Mouse sample values were adjusted for recovery.

EXPERIMENT 2-C

HPLC-CoulArray analysis of unconjugated and conjugated BPA. Two volumes of cold absolute ethanol were added to serum. Precipitated proteins were pelleted at 4^{o}C by centrifugation for 15 minutes at 3,000 *g*. The supernatant was brought to 600 µl using High Performance Liquid Chromatography (HPLC)-grade water (Fisher Scientific) and passed through a C18 Sep-Pak SPE cartridge (Waters). Sep-pak cartridges were pre-washed with 15 ml methanol to remove potential BPA contamination; prior tests had determined that BPA leakage was variable, but that the highest levels seen ere removed by this pretreatment. The SPE eluate was dried down under nitrogen, and then reconstituted in 50% methanol for HPLC separation. Conjugated BPA (glucuronidated and sulfated forms) was determined using the same sample preparation after treatment of 100 µl aliquots of serum overnight with ß-glucuronidase/aryl sulfatase (Sigma). Concentrations of BPA in sample extracts were determined by HPLC with an ESA CoulArray 5600 detector. Separation was performed on a reverse-phase 250 mm Prodigy C18 column (Phenomenex), with a mobile phase of 36:24:40 acetonitrile: methanol: 0.05 M sodium acetate buffer (pH 4.8), and with the CoulArray cell potentials set at 325, 400, 720 and 875 mV. The limit of detection under these conditions was 9 ng/ml. Extraction efficiency was assessed using mouse serum samples spiked with 5 ng BPA, extracted as described above; recoveries averaged 89.97%.

REFERENCES

Taylor JA, Welshons WV, Vom Saal FS. 2008. No effect of route of exposure (oral; subcutaneous injection) on plasma bisphenol A throughout 24h after administration in neonatal female mice. Reprod Toxicol 25(2): 169-176.